THE LOCATIONS OF COLLAGENS WITH DIFFERENT THERMAL STABILITIES IN FIBRILS OF BOVINE RETICULAR DERMIS

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The techniques of differential scanning calorimetry and electron microscopy were combined to locate collagens with different thermal stabilities in bovine dermis. When calfskin was heated at 1.25°C/min, denatured cores developed in the fibrils at 65°C, leaving native-banded sheaths. Coincident with the initiation of shrinkage and loss of molecular orientation at 68°C, the sheaths of the fibrils began to be denatured at distributed sites along the fibrils. At 80°C the collagen lost its organized fibrillar structure. When thermally labile crosslinks had been stabilized by reduction with borohydride, an endotherm lying above 66°C was suppressed, with proportional lowering of the total enthalpy change, and a fibrous texture revealing a helical subfibrillar structure remained. The three populations of collagen are located in the same fibrils. One, located in the cores of the fibrils, is half denatured at 68°C. Another, established by crosslinks, is competent to sustain the regular appearance of fibrils even after 56% of the collagen in them has been denatured. This population is located as sheaths at the peripheries of the collagen fibrils. A third, denaturing below 59°C, is codistributed with one or both of the two others.

KEYWORDS: Collagen, stability, differentiate, scanning calorimetry, electron microscope, fibrils, dermis

INTRODUCTION

The process by which the type-I collagen fibril is formed *in vivo* or *in vitro* remains a matter of considerable uncertainty. One method of investigation has been to study its substructure, hoping there to discern the trace of the assembly process. One then tries to explain such features as the intermolecular stagger, suggesting assembly by 4-D[2] or 1-D[3] staggered molecules, or the laminae parallel to the fibril axis,[1] suggesting lateral assembly of sheets of procollagen segment long-spacing (SLS) forms.[4] Carrying this type of analysis to a stage of fibril development later than the assembly, we report here a radial inhomogeneity of the fibrils in bovine reticular dermis, not previously investigated, that may be due to the mechanism of intermolecular crosslinking. In these experiments we developed the substructure by partial thermal denaturation so that we could observe it by means of transmission electron microscopy.

METHODS

Skin from the posterior dorsal region of 6 mo calf or of mature (18 mo) steer was frozen within 2 hours of slaughter and stored at −35°C. The reticular dermis was isolated by dissection while the specimen was kept partially frozen, and the sample was kept on ice for less than 4 hours thereafter before the DSC scan.

Differential scanning calorimetry (DSC) on fully hydrated calfskin was performed with a Perkin-Elmer DSC-2 instrument and hermetically sealed aluminum capsules under flowing nitrogen. The 18 mg wet samples were scanned at 1.25°C/min, with water in the reference capsule. When we investigated the reduced steerhide, however, we were constrained to use a Perkin-Elmer DSC-1 calorimeter, and so had to obtain the thermogram at 10°C/min; the endothermic peak at 63°C remained at that temperature for both rates. While previous observations[5] would have led us to expect a 6°C increase in the apparent transition temperature at the higher heating rate, those observations had been carried out on samples twice the size of ours in suspensions containing one tenth the concentration of collagen. Further, the collagen in our experiments was natural, rather than purified or reconstituted, affecting the stability of the fibrils (higher denaturation temperature) and the composition of the melt above the melting temperature. For neither a scanning rate of 1°C/min nor 10°C/min can the system be at equilibrium, at least for the unmelted collage.[6] The temperature scales of the instruments were calibrated with the melting points of 99.999% naphthalene (352.9 K) and indium (429 K); the sensitivities, with the enthalpy of 99.9% benzoic acid (141.9 J/g). After each temperature scan, the sample (ca. 5 mg) was removed from its capsule, dried at 110°C for 4 hours, weighed, and analyzed for hydroxyproline.[7]

Gaussian curves were fitted to experimental endotherms by the Gauss-Newton iteration technique,[8] using a Modcomp Classic 7861 computer with the program ABACUS D.2 developed by the ERRC computer center. Data from recorder charts were first digitized by means of a digitizing pad.

When we wanted to observe the structure of the collagen residue at any temperature, we interrupted the scan at that temperature, quickly removed the capsule and quenched it with dry ice, opened it, macerated the sample with a scalpel, and dropped the fragments into the glutaraldehyde fixative described below.

The unsaturated crosslinks, condensation products of lysine and allysine, were chemically reduced by cutting 160 mg hide, containing about 40 mg collagen, from an 18 mo old steer into 80 μm slices, suspending them in 50 ml of 50 mM phosphate at pH = 7.4, and adding 2 mg solid sodium borohydride. After 90 min at room temperature the tissue was removed to water at pH = 3 for 30 min at 4°C and then was resuspended in the cold neutral buffer. The treatment with low pH, used to quench the borohydride reagent, did not appear to affect the thermogram of the final product, implying that the reduction reaction removed nearly all labile crosslinks. Mature steerhide was used in this experiment in order to demonstrate the more clearly the suppression of the high-temperature endotherm, which is more prominent in mature hide than in calfskin.

Shrinkage measurements were made on $5 \times 5 \times 40\,mm^3$ strips of hide mounted in an apparatus described previously.[9] The lower end of the sample strip was

fixed; the upper end was clamped to the end of a counterbalanced lever arm, which moved vertically. The moving clamp was attached to the core of a linear variable differential transformer, which sent the AC position signal to the Sensotek demodulator and amplifier. Temperatures were measured with a thermistor. The two DC signals were digitized, stored, and later analyzed and plotted with a Keithley Soft500 DAS system and an IBM PC microcomputer. Loaded with a 20 g weight, the sample was heated in water at 1.25°C/min from 35°C to 100°C while its length was recorded at 0.01°C intervals.

The birefringence of a single fiber bundle, in a 40 μm section mounted in water on a microscope slide, was measured with a Babinet compensator mounted on a microscope (Gaertner Scientific Co.) with a 10-X objective, during heating at 1°C/min with a Koeffler hot stage.

Samples removed from the calorimeter capsules were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, with or without 1% tannic acid, at pH = 7.4 for 18 hours at 22°C. After having been rinsed in this buffer and in water, they were stained *en bloc* with 1% aqueous uranyl acetate or ruthenium red solution for 2 hours, placed in a water-soluble melamine resin, Nanoplast™ (Polysciences, Inc., Warrington, PA 18976), and cured in a desiccator at 40°C for 48 hours and then at 60°C for 48 hours. The micrographs shown in this paper are all of samples that were prepared with tannic acid-uranyl acetate. When conventional epoxy was used for embedding, thin sections of thermally denatured samples sank in the trough of the diamond knife during microtomy, but the melamine resin[10] yielded uniformly good results. Micrographs of 60 nm sections were obtained with a Zeiss EM10B electron microscope operated at 60 kV.

RESULTS

A typical thermogram for the reticular dermis of calf skin is shown in Fig. 1. Three endotherms are apparent, with peaks at 56.5°C, 63°C, and ca. 75°C (pronounced shoulder), respectively. The first peak was not observed in the hide of mature cattle;[11] the others were. The third was sensitive to low pH[11] and to reduction of the various lysine and allysine condensation products with borohydride (Fig. 1b). The second endotherm persisted as a sharp peak after various treatments including borohydride reduction (Fig. 1b), aging, tanning, which caused the temperature at the peak to increase, and acid or base, which caused it to decrease (11).

As shown in Fig. 1a, the thermogram of natural dermis was represented by the sum of three Gaussian functions well enough to allow us to determine empirically the contribution of each component to the total enthalpy. The total energy absorbed over all three steps is 54.3 J/g of collagen, the same as that found for collagen in other tissues or physical states.[12,5] We took the agreement as evidence that the specific enthalpy change is the same for the overall process as for the process associated with each component peak and that it is due to denaturation of dermal collagen molecules.[11] If this is the case, the three contributions to the enthalpy also represent the relative masses of the three collagen populations: 13%, 47%, and 40%, respectively.

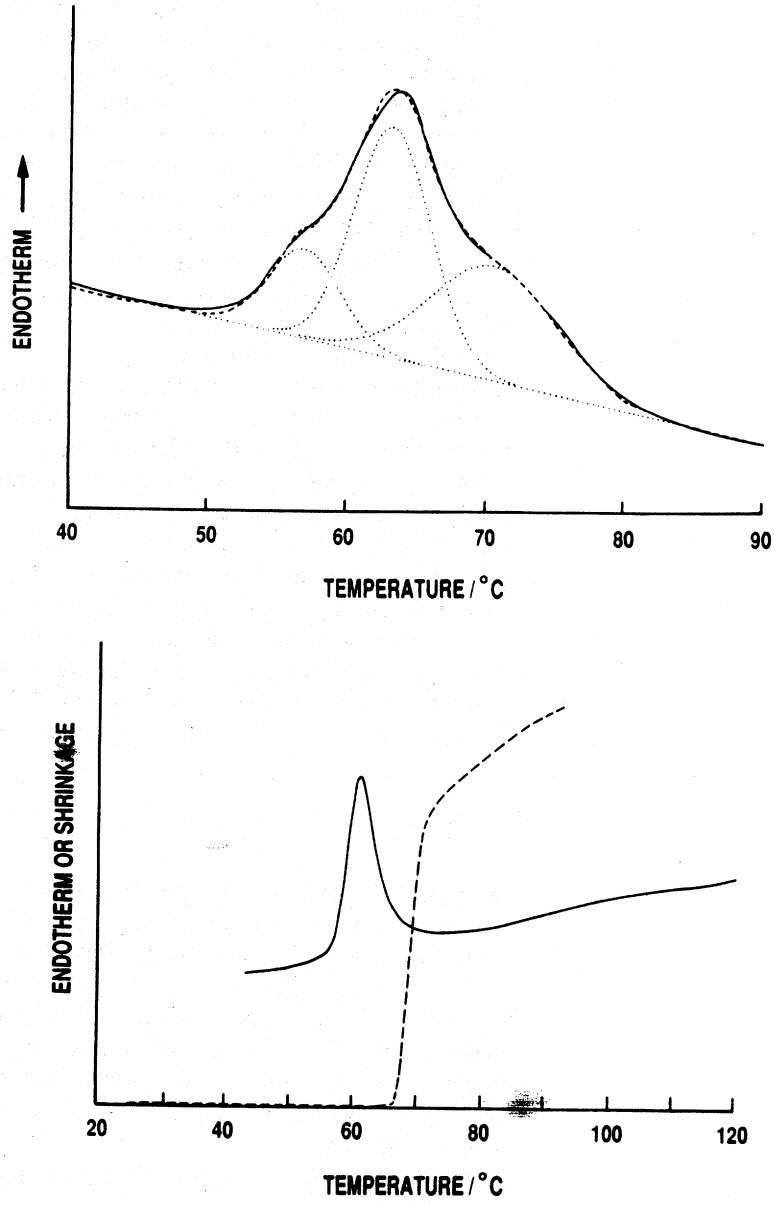

FIGURE 1a. Endotherm of collagen in calf dermis (____), showing also three Gaussian components (.....) and their resultant (– – – –). The whole ordinate spans 0.2 mW. (b) Endotherm and thermal shrinkage of mature dermis reduced with borohydride. Whole ordinate spans 10 mW or 25% shrinkage.

Fig. 2 compares the thermogram with the birefringence and with the thermal shrinkage of the same tissue heated at the same rate in water. There was no shrinkage or loss of birefringence until the tissue had been heated to 68°C, when, according to the area under the thermogram, 56% of the collagen had already been denatured in the native dermis (Fig. 1a) and from 63% to 86% in the reduced tissue (Fig. 1b), varying from sample to sample.

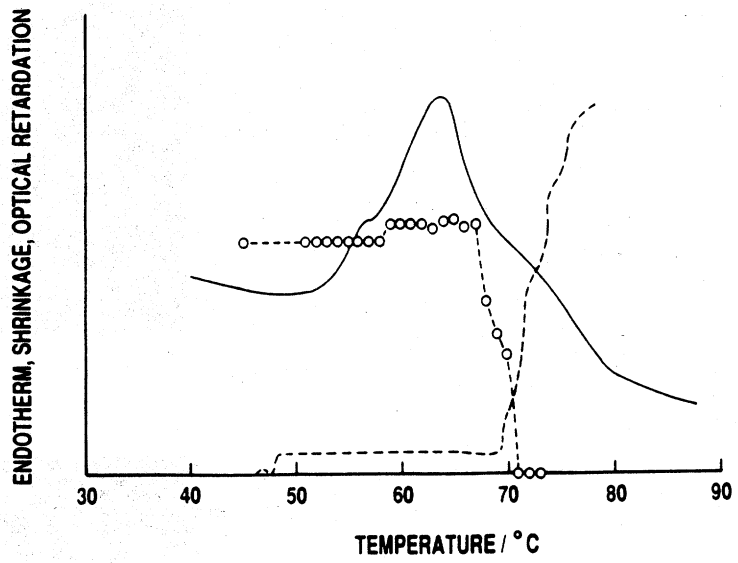

FIGURE 2 Endotherm (——), thermal shrinkage (– – – –), and change in birefringence (o...o...o) of collagen in calf dermis during thermal denaturation. Whole ordinate spans 0.2 mW, 30% shrinkage, or 0.5 wavelengths.

Samples were taken from the calorimeter at selected temperatures and quenched for observation with the electron microscope. The rationale for choosing these particular temperatures can best be inferred from Fig. 1a, where 59°C, 66°C, 69°C, and 80°C mark points where the denaturation processes seemed to change. At 65°C the second process was being completed but the sample had not yet shrunk, whereas at 68°C the sample had begun to shrink but the third process was still fully underway.

Embedded in melamine resin and stained with tannic acid-uranyl acetate as described in METHODS, the unheated fibrils in transverse section appeared against a light background and had lightly stained interiors (Fig. 3a); the longitudinal sections (Fig. 3b) had positively stained Schmitt-Gross bands.[14] As can be seen in Figs. 3a and 3b, the edges and bands of the fibrils were well defined. Any changes that occur when the dermis was heated through the first peak to 59°C were not evident by direct examination in the electron microscope, as shown in Fig. 4.

After heating through the second peak, however, to 65°C, dark central cores appeared in the transverse sections (Fig. 5a). These cores coincided in their locations with dark streaks on the axes of the fibrils in the longitudinal sections of Fig. 5b. These streaks and their correspondence with the cores are shown more clearly in Fig. 5c, which was obtained from a sample heated separately at a higher rate, which did not alter the thermogram qualitatively. The dark cores of Fig. 5a would have been expected to show as central dark streaks against the cross-bands in Fig. 5b because the sections were 60 nm thick, more than half the diameter of the fibrils. Thus, dark cores were observed to be superposed on the banding pattern of the fibril sheath. Fig. 5 shows then that the fibrils in the sample that

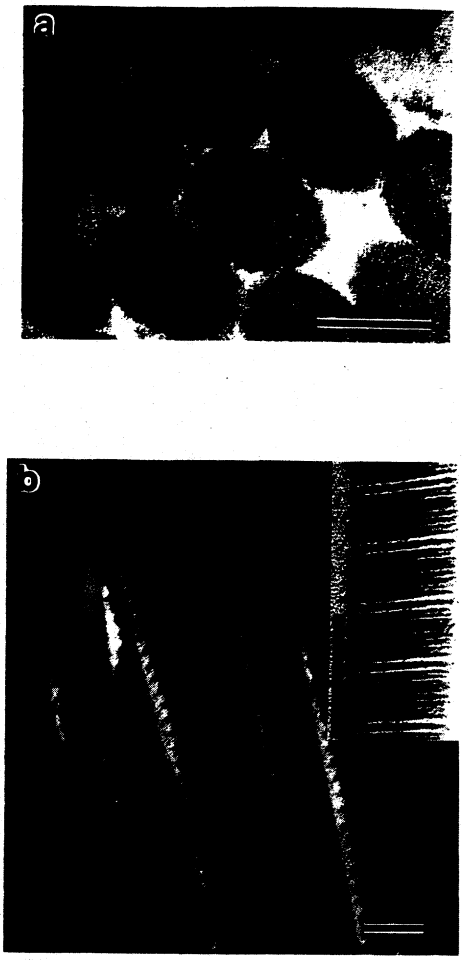

FIGURE 3 Collagen fibrils in unheated calf-skin dermis. (a) transverse section. Bar, 100 nm; (b) longitudinal section. Bar, 200 nm. Inset: bar, 100 nm.

had not yet undergone thermal shrinkage, but had been 55.6% denatured (from data in Fig. 1a), had sheaths with obvious intermolecular order still preserved.

A striking feature of the heated samples in Fig. 5 was the dark background. Although samples heated to 65°C appeared to be either negatively stained or photographed in reverse contrast in their micrographs, the normal, positively stained Gross-Schmitt bands showed them to be neither. This did not seem to be an observational photographic artifact: neither unusual microscope settings nor photographic processes were used to develop these bands at their usual intensity and contrast. There seems to have been a stain-binding material in these spaces, at least after the heating. Even before being heated this material might also have been present in a latent, unstainable form, perhaps undergoing a physical change under heating which caused it to bind uranyl ions. Studies under way to identify this interfibrillar material and also the material in the cores show it to vanish after

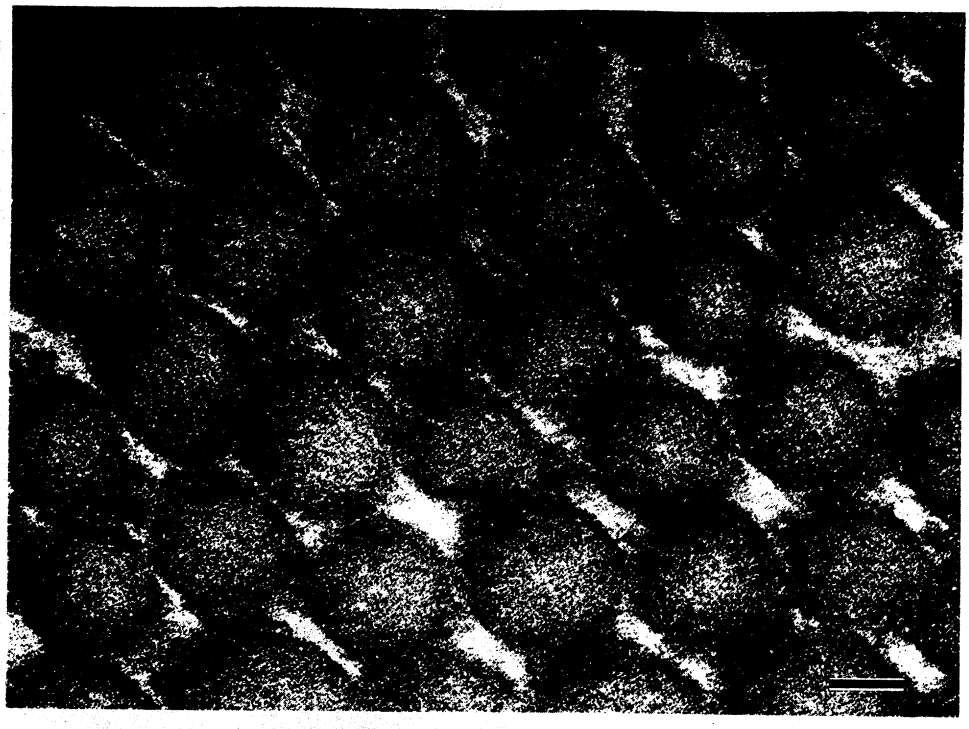

FIGURE 4 Collagen fibrils in calf-skin reticular dermis heated at 1.25°C/min to 59°C. Bar, 100 nm.

treatment with trypsin. Unlike the stands[13] observed to be associated with the d-bands[14] of the fibrils when the tissue is embedded in epoxy resin, this material was dispersed. We noticed that it was stained with either uranyl acetate or ruthenium red even without tannic acid (micrograph not shown). Tannic acid has been found to enhance the binding of metal cations to polysaccharides.[15] The behavior then was consistent with the properties of a small proteoglycan, perhaps proteodermatan sulfate.[16] It appears (Figs. 4 and 5) to have been stained more deeply and to have been more dispersed after it had been heated, perhaps because of its own denaturation. Its presence in the cores was unexpected and unexplained, but ruthenium-red stained cores have been shown in collagen fibrils of bovine heart valves in epoxy resin,[17], and uranyl acetate-lead citrate staining cores, in fibrils of quick-frozen and freeze-substituted dermis in epoxy resin.[18]

The sheaths were first observed to have become denatured along the lengths of the fibrils at 68°C (Fig. 6), at which the fibers had just become isotropic (Fig. 2) and the sample had begun to shrink. The sheaths, like the cores, appeared to be transformed into a dark-staining amorphous material. This denaturation of the sheaths appeared to be progressive, since at 80°C, at which denaturation was virtually complete and shrinkage was severe, fibril definition and banding were not observed (Fig. 7a). In the dermis that has been reduced with borohydride, however, the fibrils were preserved even when heated to 120°C, although the

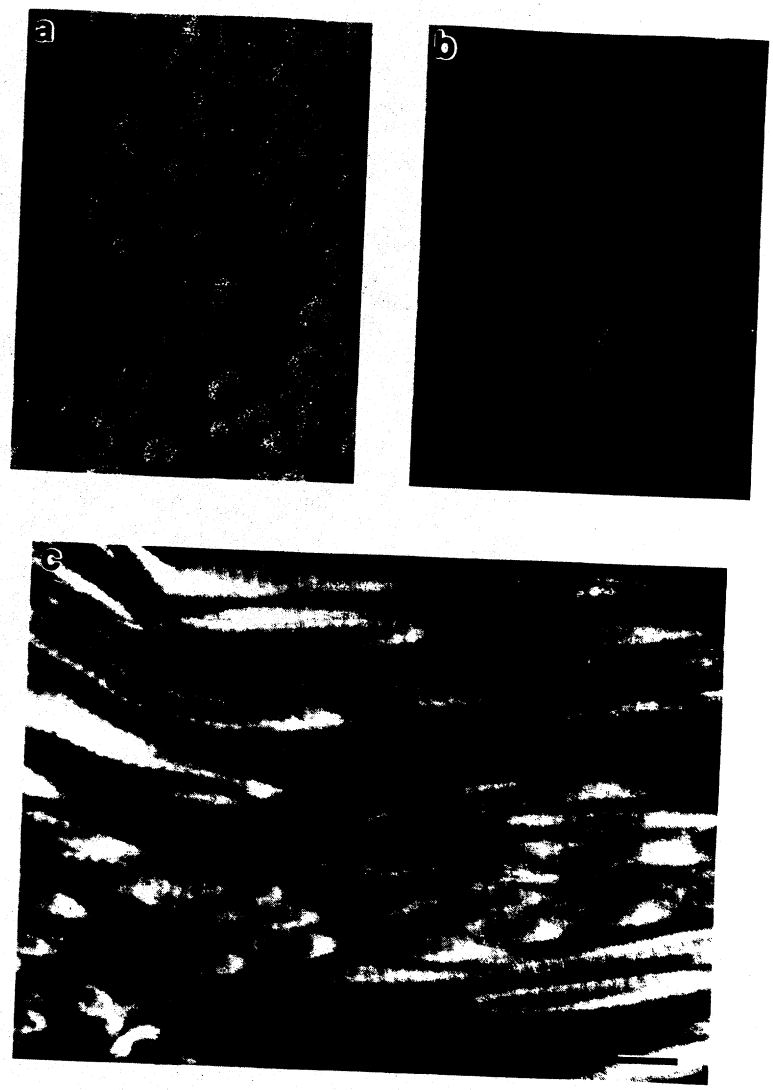

FIGURE 5 Collagen fibrils in calf-skin reticular dermis heated to 65°C. (a) transverse sections heated at 1.25°C/min; (b) longitudinal sections heated at 1.25°C/min; (c) longitudinal sections heated at 10°C/min. Bar, 200 nm.

lateral register of the molecules, indicated by the bands, was lost (Fig. 7b). Fibrils have expanded in diameter and appear to have coiled into helices.

DISCUSSION

The endotherm of calf skin in Fig. 1, with three components, was very similar to those reported for the skin of young rats.[19] Peak 1 has been shown to be related to a class of collagen, which comprised 13% of the total in the calf reticular

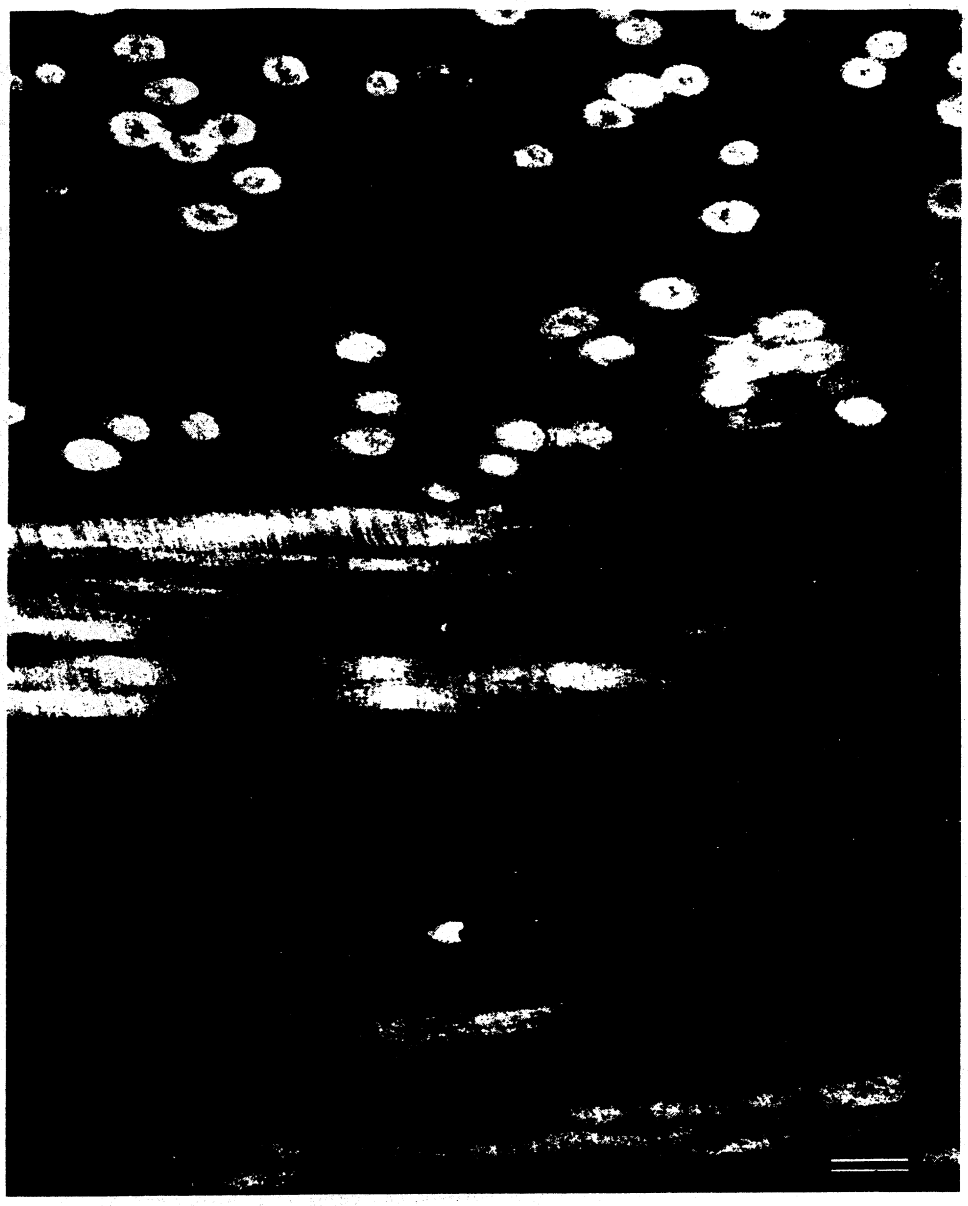

FIGURE 6 Collagen fibrils in calf-skin reticular dermis heated at 1.25°C/min to 68°C. Bar, 200 nm.

dermis and was not crosslinked, since it occurs only in dermis of young or lathyritic rats.[19] On quenching after this denaturation step, however, we observed no change in the morphology of the fibrils (Fig. 4). Clearly, this most labile fraction of collagen was not segregated in any one part of the fibrils; it might not have been distributed uniformly, but could not occur by itself.

The core regions of the fibrils were comprised of material that was unstable at

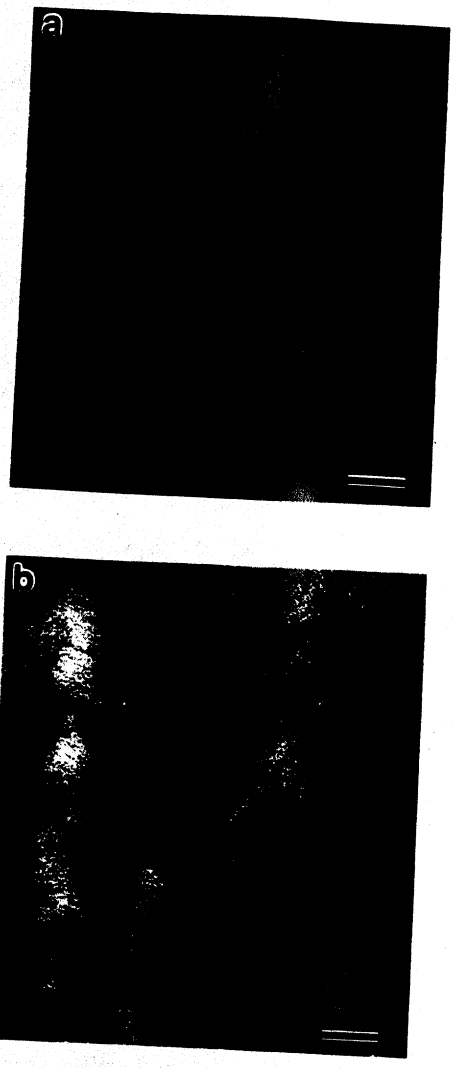

FIGURE 7 Collagen fibrils in heated reticular dermis. (a) Native calf dermis heated at 1.25°C/min to 80°C; (b) mature dermis, reduced with borohydride and heated at 10°C/min to 120°C. Bar, 200 nm.

temperatures between 52°C and 72°C, the region of the second denaturation peak (Fig. 1). Unlike the first peak, this one appeared in the skin of both young and mature animals[11,19] and thus was not due to recently synthesized collagen. During this denaturation step, the shape of the fibril was preserved by the stable sheath, in which lateral order among the molecules was not disturbed. That is, there was no macroscopic shrinkage, no change in form birefringence, no significant change in fibril diameter, nor loss of cross-banding.

Figs. 5 and 6 show that the most stable collagen fraction in the dermis comprised a peripheral sheath about each fibril. The presence of such stable material was also revealed in collagen denaturation endotherms of rat skin,[19] where it was

identified as collagen with thermally labile crosslinks.[19-21] It may be noteworthy that a peak or shoulder on the same temperature interval (75°C) was completely absent in the endotherm of reconstituted collagen fibrils,[5] which would have contained fewer crosslinks. Labile crosslinks were shown to decompose on the same temperature interval as the third endotherm, allowing collagen helices to be denatured.[20] We found that during this process the fibrils swelled, the edges were lost, and, as also shown before,[20,21] the sample shrank. After stabilization of the molecules by chemical reduction of the crosslinks, the third endotherm was spread out and was greatly diminished (Fig. 1b), and the fibrils persisted in Fig. 7, although they swelled about 50% in diameter, and their edges became diffuse. Reduction, however, did not eliminate thermal shrinkage (Fig. 1b) nor loss of ordered molecular packing.

The helicoid filamentous substructure, developed by thermal denaturation of the reduced collagen (Fig. 7b), resembled that seen previously in tissue collagen denatured by 4M guanidinium chloride.[22]

The crosslinked collagen molecules were evidently segregated into circumfibrilar sheaths as we show here because they must have reacted extracellularly with lysyl oxidase, which, however, could not easily diffuse into the fibril itself. Its reaction product was therefore localized at the periphery of the fibril, where it subsequently formed the crosslinks involving allysine and lysine. This reaction must have occured after the fibrils organized laterally and thickened. We also found DSC evidence that this sheath-core substructure persists even as the animal matures, more stable crosslinks evolving in the sheath but not in the core, with preservation of the constituent collagen in the native state at temperatures up to 110°C in reticular dermis of 18 mo old steers.[11]

Since the crosslinks are segregated into half the collagen of the dermis, their local density is at least twice the average concentration. This concentration should be considered in trying to relate crosslink concentrations to mechanical properties, remodelling, swelling, and industrial tanning of this tissue.

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REFERENCES

1. D. S. Hulmes, J-C. Jesior, A. Miller, C. Berthet-Colominas, and C. Wolff, *Proc. Nat'l. Acad. Sci. (USA)*, **78**, 3567–3571 (1981).
2. F. H. Silver, *J. Biol. Chem.*, **256**, 4973–4977 (1981).
3. G. C. Na, L. J. Butz, D. G. Bailey, and R. J. Carroll, *Biochemistry*, **25**, 958–966 (1986).
4. D. J. S. Hulmes, R. R. Bruns, and J. Gross, *Proc. Nat'l Acad. Sci (USA)*, **76**, 313–317, (1979); ibid., **80**, 388–392 (1983).
5. D. G. Wallace, R. A. Condell, W. Donovan, A. Paivinen, W. M. Rhee, and S. B. Wade, *Biopolymers*, **25**, 1875–1893 (1986).
6. P. J. Flory and R. R. Garrett, *J. Am. Chem. Soc.*, **80**, 4836–4845 (1958).
7. B. R. Switzer. and G. K. Summer, *Anal. Biochem.* **39**, 487–491 (1971).
8. N. Draper and H. Smith, *Applied Regression Analysis*, Wiley, New York (1966), pp. 263–304.
9. M. S. Sacks, P. L. Kronick, and P. J. Buechler, J. Food. Sci. (In press).
10. C. Westphal and D. Frosch, J. Ultrastructure Res. **88**, 282–286 (1984).
11. P. L. Kronick and P. R. Buechler, *J. Am. Leather Chem. Assn.*, **81**, 213–220, (1986).
12. P. E. McClain and E. R. Wiley, *J. Biol. Chem.*, **247**, 692–697 (1972).

13. R. D. Young, *J. Cell Sci.*, **74**, 95–104 (1985).
14. F. O. Schmitt and J. Gross, *J. Am. Leather Chem. Assn.*, **43**, 658–675 (1948).
15. C. T. Singley and M. Solursh, *Histochemistry*, **65**, 93–102 (1980).
16. R. K. Chopra, H. Pearson, G. A. Pringle, D. S. Fackre, and P. G. Scott, *Biochem. J.*, **232**, 277–279 (1985).
17. K. Nakao and R.I. Bashey, *Exp. Mol. Pathol.*, **17**, 6–13 (1972).
18. F. Mallein-Gerin and R. Garrone, *Int. J. Biol. Macromol.*, **8**, 121–123 (1986).
19. F. Flandin, C. Buffevant, and D. Herbage, *Biochim. Biophys. Acta*, **791**, 205–211 (1984).
20. J. C. Allain, M. Le Lous, S. Bazin, A. J. Bailey, and A. Delaunay, *Biochim. Biophys. Acta*, **533**, 147–155 (1978).
21. M. Le Lous, J. C. Allain, L. Cohen-Solal, and P. Maroteaux, *Conn. Tiss. Res.*, **11**, 199–206 (1983).
22. A. Ruggieri, F. Benazzo, and E. Reale, *J. Ultrastruct. Res.* **68**, 101–108 (1979).